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14. ABSTRACT

We have made progress in characterizing Rheb2, examining effects of the TSC/Rheb/mTOR signaling on cell cycle progression, investigating mechanism of activation of mTOR and examining consequences of the activation of the TSC/Rheb/mTOR signaling pathway. We have shown that the TSC/Rheb/mTOR signaling pathway affects nuclear translocation of a Cdk2 inhibitor, p27. Novel activating mutations of mTOR were identified. Consequences of the activation of the TSC/Rheb/mTOR signaling pathway on cell physiology are being investigated. Our study makes significant contribution to understand how the TSC/Rheb/mTOR signaling pathway is regulated. Our investigation into cellular consequences of the activation of this pathway is important in understanding tuberous sclerosis.

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Introduction

This report covers the period December 15, 2005 – December 14, 2006. Tuberous sclerosis is caused by mutations in the *Tsc1* or *Tsc2* gene. Products of these genes form a complex that acts as a negative regulator of Rheb GTPase. Rheb is an activator of mTOR. Thus, one of the major problems with tuberous sclerosis is that the Tsc/Rheb/mTOR signaling pathway is over-activated. Our research is focused on understanding how this signaling pathway is regulated and what the consequences of alteration of this signaling pathway are. We have made progress in experiments outlined in the Statement of Work. In addition to characterizing Rheb2, we have characterized altered cell cycle progression in the *Tsc*-null MEFs. We have generated novel mTOR mutants, important reagents to over-activate the Tsc/Rheb/mTOR signaling.

Summary of progress

Tuberous sclerosis is caused by the loss of Tsc1/Tsc2 complex that acts as a negative regulator of Rheb GTPase (Aspuria and Tamanoi, 2004). This results in overactivation of the Rheb/mTOR signaling pathway. Our overall aim is to understand the consequences of altering this signaling pathway. During the current funding period, we have made progress in this pursuit by carrying out the following experiments.
(i)We have further characterized Rheb2 protein. (ii) We have elucidated how the overactivation of the Tsc/Rheb/mTOR signaling results in altered cell cycle progression. (iii) We have generated constitutive active mutant forms of mTOR that can bypass amino acid requirement for their activation. Effects of the expression of these mutants have been investigated.

Detailed description of accomplishments

1. To generate Rheb1 and Rheb2 knockout mice

We continued our effort to generate knockout mice. To accomplish this, we consulted with Dr. Michele Musacchio at the University of California Irvine. The targeting constructs for Rheb1 and Rheb2 were electroporated into mouse ES cells and the cells with correct chromosomal replacement were screened by Southern hybridization. After extensive screening of ES cells, we failed to identify knockout ES cells for either Rheb1 or Rheb2.

2. To characterize Rheb2

Previously, we have been successful in raising a polyclonal antibody against Rheb2 (RhebL1). This antibody detects mouse Rheb2 but not mouse Rheb1. It does not recognize human Rheb2. We have continued to characterize Rheb2 using this antibody. Mouse tissue samples (brain, heart, kidney, liver, testis and muscle) were used to examine tissue expression of Rheb2. Our results (Figure 1) show that Rheb2 is highly expressed in the brain. While Rheb1 is expressed ubiquitously, Rheb2 appears to exhibit tissue specific expression. Non-ubiquitous expression of human Rheb2 was recently reported (Yuan et al., 2005; Saito et al., 2005).

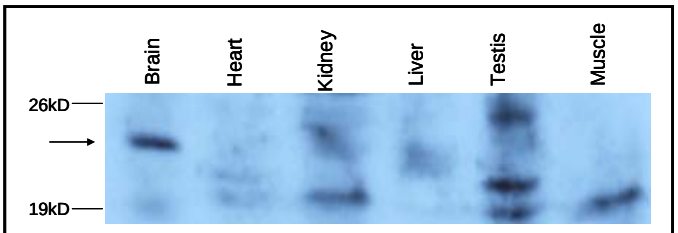

Fig.1. Detection of endogenous Rheb2 in mouse tissues. Rheb2 antibody was used to examine level of endogenous Rheb2 protein in various mouse tissues.

3. To investigate the mechanism of regulation of cell cycle progression by the TSC/Rheb/mTOR signaling pathway

The loss of TSC leads to the activation of Rheb/ mTOR signaling resulting in altered cell growth. We have previously reported using *Drosophila* tissue culture cells that the TSC/Rheb/mTOR signaling pathway plays critical roles in cell cycle progression; inhibition of this signaling leads to cell cycle arrest at the G1 phase (Patel et al., 2003). In the course of this grant, we found that the loss of TSC leads to altered cell growth in mammalian cells. This was shown using MEFs derived from *Tsc*-null mice. These cells do not respond to serum starvation and continue growing even in the absence of serum. Furthermore, they are not cell cycle inhibited after reaching high density.

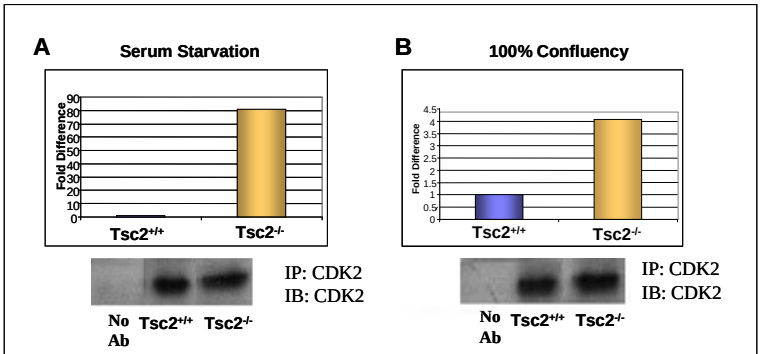

Fig.2. Cdk2 is constitutively activated in Tsc2-null MEFs. Kinase activity of Cdk2 immunoprecipitated from Tsc2-/- or the control +/+ cells after serum starvation (A) or after reaching high density was examined. The lower panels show that comparable amounts of Cdk2 were recovered in immuneprecipitates.

Cell cycle progression from G1 to S is regulated by the activity of Cdk2. We examined Cdk2 activity by immunoprecipitating Cdk2 and assaying kinase activity by using histone H1 as a substrate. This study showed that Cdk2 activity remains active even after serum starvation in the *Tsc2*-null MEFs (Figure 2A). Similarly, Cdk2 activity remains high after the *Tsc2*-null MEF cells reached high confluency (Figure 2B). In contrast, the level of Cdk2 is low in the control parental MEFs after serum starvation.

To gain insight into which cell cycle protein is affected by the alteration of the TSC/Rheb/mTOR signaling, we examined key proteins involved in cell cycle regulation such as cyclins and Cdk inhibitors. One of the consistently observed differences between the Tsc2-null MEF and control MEF concerns nuclear localization of p27. While nuclear translocation of p27 is observed after serum starvation in the control MEF, p27 was not detected in the nuclear fraction in the *Tsc2*-null MEF (Figure 3). In collaboration with Dr. Cheryl Walker (MD Anderson Cancer Center), it was shown that the nuclear translocation of p27 is dependent on its phosphorylation by AMPK. The AMPK phosphorylation sites on p27 have been identified.

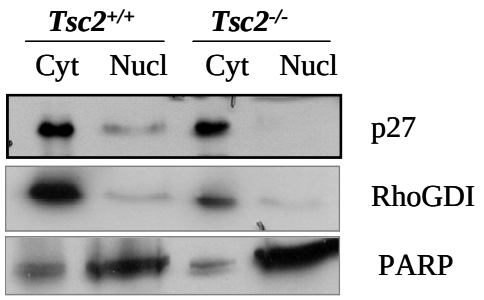

Fig.3. Nuclear translocation of p27 Is blocked in the Tsc2-null MEFs. The amount of p27 in the nuclear and cytosolic fractions was examined by Western analysis. RhoGDI and PARP are used as cytosolic and nuclear markers, respectively.

3. To elucidate mechanisms that result in the activation of the TSC/Rheb/mTOR signaling pathway

Because tuberous sclerosis arises from the activation of the TSC/Rheb/mTOR signaling, it is important to understand how this signaling pathway can be activated and what consequences activation of this signaling pathway have on cell physiology. Recently, a set of fission yeast Tor2 mutants that are constitutively active has been identified. These mutants, each having a single amino acid change, were obtained by our study on the fission yeast model system. In fission yeast, there are two Tor proteins, Tor1 and Tor2. Tor2 forms TORC1 complex together with Mip1, a fission yeast homologue of Raptor. On the other hand, Tor1 forms TORC2 complex together with Ste20, a fission yeast homologue of Rictor. Tor2 is essential for growth and is activated

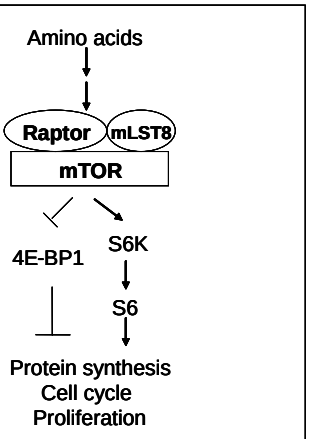

Fig.4. TORC1 complex containing Raptor and mLST8 responds to amino acids and phosphorylates S6K and 4E-BP1, leading to cell growth and cell cycle progression.

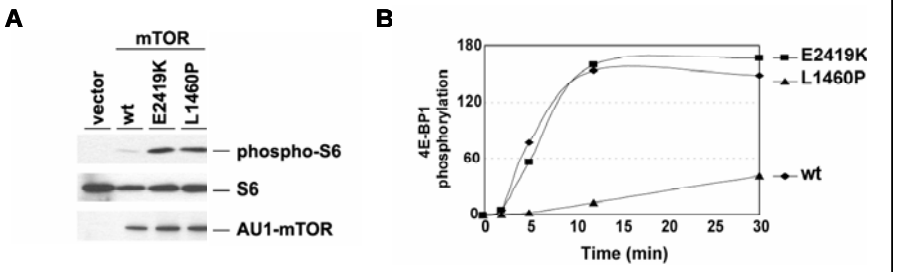

Figure 5. A: HEK293 cells exhibit low phospho-S6 level after nutrient starvation. In contrast, in cells transfected with constitutive active mTOR mutants (E2419K or L1460P), high level of phospho-S6 is detected. B: Kinase activity in vitro is decreased after nutrient starvation in cells transfected with the wild type mTOR. However, high level activity is detected in cells transfected with the constitutive active mTOR mutants.

by Rheb which is also an essential protein. We have devised screens to identify mutant forms of Tor2 that can bypass dependency on Rheb for growth. In addition, another screen based on the inhibition of mating was carried out. Altogether, twenty two different single amino acid changes were identified that confer Rheb independent growth. Interestingly, these mutations were clustered in two regions one in the FAT domain and

the other in the kinase domain. Most of the mutations we identified occur on residues that are conserved in mTOR.

In the study supported by this grant, we have introduced representative mutations into mTOR. A representative mutation from each domain, the FAT domain and the kinase domain, was selected. These mutant mTOR proteins were expressed in HEK293 cells and their effects on amino acid dependent growth were examined. As shown in Figures 4 and 5, mTOR activity is dependent on the presence of amino acids. Therefore, mTOR activity is low when cells are amino acid starved. This is seen in Figure 4 that investigated mTOR activity after nutrient starvation by examining phosphorylation of S6 (A) or carrying out kinase activity of mTOR immuneprecipitates (B). While transfection of the wild type mTOR did not rescue nutrient starvation, significant level of mTOR activity was detected when constitutive active mTOR mutants were transfected. Therefore, these mutants confer amino acid independent growth.

Two types of mTOR complexes are present in mammalian cells. A complex termed mTORC1 contains mTOR, Raptor and mLST8 and is involved in growth control mediated by the stimulation of protein synthesis. Another complex mTORC2 contains mTOR, Rictor and mLST8 and is responsible for the phosphorylation of Akt. We examined whether our mutations also affect mTORC2 activity. The results obtained suggest that mTORC2 is unaffected by the activating mutations.

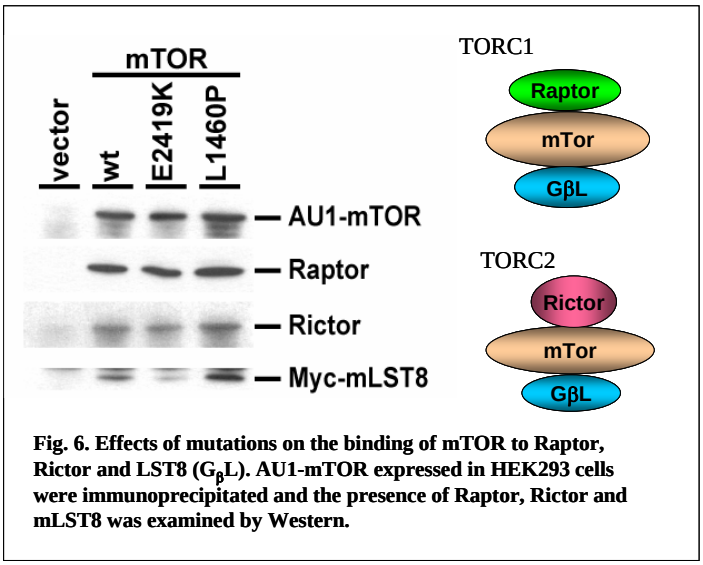

Fig. 6. Effects of mutations on the binding of mTOR to Raptor, Rictor and LST8 ($G_{\beta}L$). AU1-mTOR expressed in HEK293 cells were immunoprecipitated and the presence of Raptor, Rictor and mLST8 was examined by Western.

One possible mechanism for mTOR activation by the activating mutations is that the binding of mTOR associated proteins will be altered. To examine this point, mTOR was immunoprecipitated and the presence of associated proteins, Raptor, Rictor and mLST8 was examined. We find comparable levels of associated proteins with the constitutively active mTOR compared with the wild type protein (Figure 6). Thus, the mutations do not affect overall structure of the mTOR complex.

Another important finding we made is that these mutations exert dominant effects. It is known that mTOR exists as a dimer. This was demonstrated by using two different mTOR tagged with different tags, AU1 and FLAG (Figure 7). These mTOR are co-expressed. When AU1 mTOR was immuno-precipitated, we found that FLAG-mTOR also came down in the immuneprecipitates, suggesting that a dimer form of mTOR is present.

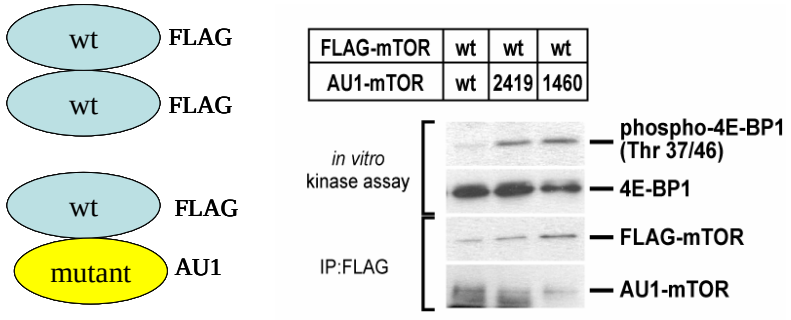

Fig.7. Left: schematic representation of dimer forms of mTOR. Right: FLAG-mTOR and AU1-mTOR were co-expressed in HEK293 cells. The resulting mTOR complex was immunoprecipitated using Anti-FLAG antibody. Detection of AU1-mTOR in the immune-Precipitates suggests heterodimer formation. Kinase activity of mTOR is constitutively active with the heterodimers.

We then constructed AU1 tagged mutant mTOR and co-expressed it with FLAG tagged wild type mTOR. When FLAG-tagged mTOR was immunoprecipitated, we observed coprecipitation of AU1-mTOR. When the activity of this complex was examined, we found that it was active even in the presence of mutant mTOR. These results shown in Figure 7 provide evidence that the effects of

mutations in mTOR can be dominant. This observation is important, as homozygous mutations could result in constitutive activation of mTOR. It will be interesting to actually identify mTOR mutations in tumor samples. Experiments are ongoing to test this possibility.

Another important observation we made concerns rapamycin that is evaluated in clinics as anticancer drugs. As shown in Figure 8, rapamycin inhibited constitutively activated mTOR. In this experiment, the wild type and two different mutants of mTOR were transfected into cells. The cells were starved and then treated with rapamycin. Phosphorylation level of S6 was examined to assess the activity of mTOR. The results suggest that the activity of the mutant mTOR can still be inhibited by rapamycin.

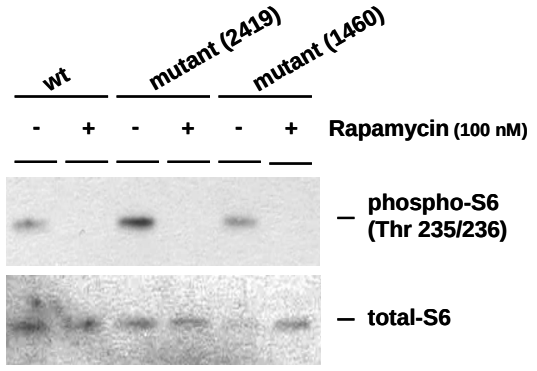

Fig.8. The activity of mTOR was examined by the phosphorylation of a downstream protein S6. The wild type as well as mutant activities were inhibited by rapamycin.

Currently ongoing projects and plans for the next funding period

We plan to continue our approach to (i) characterize Rheb2, (ii) investigate the mechanism of cell cycle progression by the TSC/Rheb/mTOR signaling, (iii) elucidate mechanism of activation of mTOR and (iv) examine consequences of activation of mTOR. One particular emphasis is to identify mTOR mutations in tumor samples. Another emphasis is to generate cell lines stably expressing constitutive active mTOR mutants. HEK293 cells were transfected with a construct to express mTOR active mutants. Transfectants were selected by G418 and single clones were isolated. Stable expression of the mutant mTOR was confirmed. We plan to carry out a systematic analysis of the cells stably expressing mutant mTOR. One of the interesting questions is to examine whether growth properties such as the ability to grow on soft agar and the ability to respond to starvation are altered. In addition, morphology of the stable cell lines will be examined. Preliminary data suggest that these clones rescue the decrease in cell size by nutrient starvation. Further experiments will elucidate the mechanism by which mTOR regulates cell morphology.

Key Research Accomplishments

(1) We examined tissue expression of Rheb2, and we found that the expression was not ubiquitous. This is different from the expression profile of Rheb1.
(2) We found that the activation of the TSC/Rheb/mTOR signaling leads to constitutive activation of Cdk2, a key cell cycle protein functioning at the G1/S phase boundary. We also found that a Cdk inhibitor protein p27 is affected by the activation of the TSC/Rheb/mTOR signaling pathway. Its translocation to the nucleus is blocked.
(3) Novel mutants of mTOR that are constitutive active have been obtained.
(4) We have shown that the expression of these mutants confers constitutive activation of mTOR even in the absence of amino acids.
(5) The activating mutants of mTOR appears not to affect mTORC2 activity.
(6) The activating mutations do not alter binding of mTOR associated proteins.
(7) The activating mTOR mutations exert dominant effects over the wild type protein.
(8) The activated mTOR mutants retain sensitivity to rapamycin.

Reportable Outcomes

Constitutive active mutants of mTOR have been generated. These will provide valuable reagents for the study of the Tsc/Rheb/mTOR signaling.

Publication

Urano, J., Sato, T., Matuso, T., Otsubo, Y., Yamamoto, M. and Tamanoi, F. (2007) Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mTOR signaling in mammalian cells. *Proc. Natl. Acad. Sci. USA* 104, 3514-3519.

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Conclusions

During the current funding period, we have accomplished:

1. Further characterization of Rheb2.
2. Elucidation of the effects of the activation of the TSC/Rheb/mTOR signaling on cell cycle progression.
3. Established the significance of p27 in the cell cycle effects of the TSC/Rheb/mTOR signaling.
4. Identified novel activating mutations of mTOR.
5. The activating mutations confer amino acid independent activation of mTOR.
6. The activating mutations exert dominant effects.
7. The activated mutants retain rapamycin sensitivity.

These studies should provide important insights into understanding the consequences of altering the TSC/Rheb/mTOR signaling.

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Appendix 1

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Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells

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Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells

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Edited by Peter K. Vogt, The Scripps Research Institute, La Jolla, CA, and approved December 27, 2006 (received for review September 27, 2006)

Rheb is a unique member of the Ras superfamily GTP-binding proteins. We as well as others previously have shown that Rheb is a critical component of the TSC/TOR signaling pathway. In fission yeast, Rheb is encoded by the *rhb1* gene. Rhb1p is essential for growth and directly interacts with Tor2p. In this article, we report identification of 22 single amino acid changes in the Tor2 protein that enable growth in the absence of Rhb1p. These mutants also exhibit decreased mating efficiency. Interestingly, the mutations are located in the C-terminal half of the Tor2 protein, clustering mainly within the FAT and kinase domains. We noted some differences in the effect of mutation in the FAT domain (L1310P) and in the kinase domain (E2221K) on growth and mating. Although the Tor2p mutations bypass Rhb1p's requirement for growth, they are incapable of suppressing Rhb1p's requirement for resistance to stress and toxic amino acids, pointing to multiple functions of Rhb1p. In mammalian systems, we find that mammalian target of rapamycin (mTOR) carrying analogous mutations (L1460P or E2419K), although sensitive to rapamycin, exhibits constitutive activation even when the cells are starved for nutrients. These mutations do not show significant difference in their ability to form complexes with Raptor, Rictor, or mLST8. Furthermore, we present evidence that mutant mTOR can complex with wild-type mTOR and that this heterodimer is active in nutrient-starved cells.

constitutive active TOR | FAT domain | kinase domain | mating | TORC1

Rheb comprises a unique subfamily of the Ras superfamily of GTP-binding proteins that is conserved from yeast to human and plays important roles in cell growth and cell-cycle regulation (1). We and others have shown that Rheb is an activator of mammalian target of rapamycin (mTOR) and a component of the TSC/mTOR/S6K signaling pathway that regulates protein synthesis in response to growth, energy, and nutrient conditions (1–7). mTOR exists in two distinct protein complexes: mTORC1, which consists of mTOR, Raptor, and mLST8 and is rapamycin-sensitive; and mTORC2, which consists of mTOR, Rictor, mLST8, and mSin1 and is rapamycin-insensitive. mTORC1 is involved in the regulation of translation and cell cycle, and mTORC2 is reported to be involved in actin organization and morphology (8–16). Rheb is down-regulated by a complex consisting of *TSC1* and *TSC2* gene products that act as GTPase activating proteins for Rheb (1–7). Mutations of these genes result in tuberous sclerosis complex, a genetic disorder associated with the appearance of hamartomas in the kidneys, lungs, brain, and skin (17, 18).

In fission yeast, Rheb is encoded by the *rhb1* gene and is essential for growth. Loss of Rhb1p results in small rounded cells arrested with G_1 content of DNA (19, 20). Like mammalian cells, Rhb1p is down-regulated by the Tsc1p/Tsc2p complex (21, 22). Mutations in the *tsc* genes result in a delayed response to nitrogen starvation as well as defects in amino acid uptake (21–25). The fission yeast genome encodes two TOR proteins, Tor1p and Tor2p (26, 27). Although Tor2p, like Rhb1p, is essential for growth, *tor2-*Δ cells are sterile and unable to arrest in G_1 in response to nitrogen starvation (26, 27). Tor1p also is implicated in the regulation of stress response and uptake of some amino acids (26–28). We recently have reported that fission yeast Rhb1p associates with Tor2p (25). Interaction between mammalian Rheb and mTOR also has been reported (29–31).

Because the requirement for Rhb1p for growth in fission yeast likely reflects its vital role in the activation of Tor2p, we speculated that activating mutations of Tor2p may bypass this requirement. Identification of such mutations, which are likely to be constitutive active, is of interest in gaining insight into the mechanism of activation of this kinase. In particular, this study may reveal novel involvement of specific domains in the activation of TOR. The TOR kinases are members of the phosphatidylinositol 3-kinase (PI3 kinase)-related family of kinases and have specific domains, including the HEAT repeats and the FAT, kinase, and FATC domains. Involvement of the HEAT domain in protein–protein interactions, dimerization, and membrane association has been reported (9, 32–38). To date, only one activated TOR mutant has been reported. This mutant, ΔTOR, contains a deletion of the "repressor domain," which includes AMPK (T2446) and S6K (S2448) phosphorylation sites at the C-terminal region of mTOR (39–43). In our study, we carried out a systematic analysis to uncover single amino acid changes that confer constitutive activation of TOR proteins.

We report the identification of 22 different single amino acid changes that confer constitutive activation of Tor2p. Interestingly, the mutations are clustered into two regions: the FAT domain and the kinase domain. Characterization of these mutations showed that they are able to bypass the requirement of Rhb1p for fission yeast growth. The use of these mutants allowed us to observe that Rhb1p also is required for resistance to high salt, high temperature, and toxic amino acid analogs in fission yeast. Furthermore, mTOR carrying analogous mutations exhibited nutrient-independent activity and were able to form mTORC1 and mTORC2. In addition, a heterodimer of wild-type and mutant mTOR also displayed nutrient-independent activity.

Results

Identification of Mutations in Tor2p That Can Bypass Growth Requirement for Rhb1p in Fission Yeast. Rhb1p interacts with Tor2p, and both Rhb1p and Tor2p are essential for growth (19, 20, 25, 27).

Author contributions: J.U., T.S., T.M., Y.O., M.Y., and F.T. designed research; J.U., T.S., T.M., and Y.O. performed research; J.U. and T.S. contributed new reagents/analytic tools; J.U., T.S., T.M., Y.O., M.Y., and F.T. analyzed data; and J.U., T.S., T.M., M.Y., and F.T. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Abbreviations: mTOR, mammalian target of rapamycin; RIG, Rhb1p-independent growth; FOA, 5-fluoroorotic acid.

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The article contains supporting information online at www.pnas.org/cgi/content/full/0608510104/DC1.

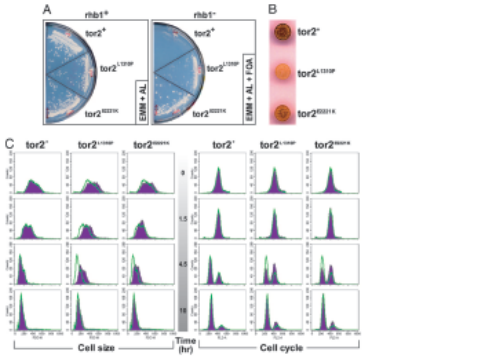

Fig. 1. Tor2p L1310F and E2221K show Rhb1-independent growth and delayed nitrogen starvation response. (*A*) Strains carrying wild-type tor2^{+} (JUp1273) or the activated mutants tor2^{L1310F} (JUp1274) and tor2^{E2221K} (JUp1275) were streaked out onto EMM + AL plates or EMM + AL + FOA plates and incubated at 30°C. EMM, Edinburgh minimal medium. (*B*) JUp1348 (tor2^{+}), JUp1350 (tor2^{L1310F}), and JUp1352 (tor2^{E2221K}) were grown to mid-log phase, washed twice with water, and adjusted to an *A*$_{595}$ of 1. Aliquots of 3 μl were spotted onto SSA plates, incubated at 25°C for 2 days, and stained by using I$_2$ vapor. Note that two sets of clones were used, and both show similar results. (*C*) JUp1348 (tor2^{+}), JUp1350 (tor2^{L1310F}), and JUp1352 (tor2^{E2221K}) were grown to mid-log phase, washed twice with water, and resuspended in SSL media. Samples were taken at the indicated times and analyzed with FACS for cell size and cell cycle. Each profile (purple) was overlayed with the profile outline for the wild-type strain (green line) at each time point.

Furthermore, loss of Tor2p function, like the loss of Rhb1p, results in small rounded cells arrested in G_1 (44, 45). Thus, it is likely that in fission yeast, Rhb1p functions to activate Tor2p. This finding raises the possibility that activating mutations in Tor2p (or another downstream factor) can confer Rhb1p-independent growth (RIG). To investigate this point, we have devised a screen to identify yeast mutants that can grow in the absence of Rhb1p [supporting information (SI) Fig. 6]. A strain (JUp1050) was constructed in which the endogenous *rhb1* gene was disrupted by using a *his3*$^{+}$ cassette and growth was maintained by using a wild-type copy of *rhb1*$^{+}$ on a *ura4*$^{+}$-based plasmid. This plasmid can be lost by counterselecting for *ura4*$^{+}$ with 5-fluoroorotic acid (FOA). JUp1050 randomly was mutagenized by methyl-nitro-nitrosoguanidine, and mutants that would grow on media containing FOA (and hence in the absence of *rhb1*$^{+}$) were isolated. To determine whether the *rig* mutation had occurred in *tor2*, we initially sequenced the entire *tor2* ORF.

Approximately 3×10^{8} cells were mutagenized and screened. After eliminating clones that still maintained the *rhb1*$^{+}$ plasmid, a single mutant strain was isolated that was able to grow in the absence of *rhb1*$^{+}$. Sequence analysis of the *tor2* ORF identified a point mutation that results in a glutamate to lysine mutation at position 2221 (E2221K) in the C-terminal half of the Tor2p kinase domain. To confirm that this mutation could confer RIG, this mutation was reintroduced into the *tor2* locus of JUp1050. This strain (JUp1261) was able to grow in the absence of *rhb1* (on FOA), confirming that this mutation in the Tor2p kinase domain confers activity independent of Rhb1p (Fig. 1*A*).

RIG Mutants of Tor2p Exhibit Decreased Mating Efficiency. Because loss of Tor2p function can induce mating (44, 45), we asked whether the Tor2^{E2221K} mutant would exhibit decreased mating. The Tor2^{E2221K} mutation was introduced into a homothallic h^{90} strain, and mating efficiency was assessed by staining with iodine that detects the increased glycogen levels in spores. As can be seen in Fig. 1*B*, we observed a notable decrease in the extent of iodine staining in the mutant. Thus, the E2221K mutation appears to decrease mating efficiency, consistent with an activation of Tor2p.

Additional Tor2 mutations (L1310P, Y1986C, I2229T, and L2233H) were identified by screening for decreased mating (decreased iodine staining) in an h^{90} strain after randomly mutagenizing the *tor2* gene. These mutations were examined for their ability to confer RIG. Fig. 1*A* shows that the strain expressing the L1310F mutant (JUp1274) grew after removing the *rhb1*$^{+}$ plasmid by FOA selection. Similar results were obtained for the other Tor2 mutants. Interestingly, when comparing the L1310P mutant with the E2221K mutant, we noticed that the strain expressing the E2221K mutant has a greater ability to suppress the Rhb1p requirement for growth (see FOA plates in Fig. 1*A*), whereas the L1310P mutant exhibits a more pronounced effect on mating efficiency (Fig. 1*B*). Thus, although RIG and decreased mating are both consequences of a single

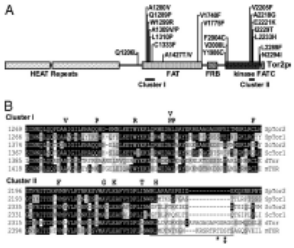

Fig. 2. Location of activated mutations in Tor2p and conservation of residues. (*A*) All identified activating mutations are indicated above the linear representation of Tor2p. Clusters I and II are indicated. (*B*) TOR protein sequences from *Schizosaccharomyces pombe* (SpTor1 and SpTor2), *S. cerevisiae* (ScTor1 and ScTor2), *Drosophila* (dTOR), and human (mTOR) were aligned with MegAlign. Residues identical or similar to SpTor2 are shaded black or gray, respectively. Mutations found in SpTor2 are indicated above the alignment. The repressor domain region of mTOR is indicated by a line under the alignment. Single and double asterisks indicate the AMPK and S6K phosphorylation sites, respectively.

activating mutation in Tor2p, each mutation may affect these two activities differently.

These Tor2 constitutive active mutants exhibit delayed nitrogen-starvation response. Because fission yeast cells respond to nitrogen starvation by arresting in G_1 as small rounded cells, nonauxotrophic strains carrying the L1310P or E2221K mutation were nitrogen-starved in SSL media (see *SI Materials and Methods*), and samples were assessed for cell size and DNA content by FACS. The results are shown in Fig. 1*C*. Forward-scatter analysis shows that both Tor2 mutants show a delay in the change in cell size compared with wild type (notably at 1.5 and 4.5 h). By 10 h, the mutant cells have decreased in size similar to wild-type cells. Analysis of cell-cycle profiles shows that the Tor2 mutants are delayed in the appearance of G_1 cells because more cells are in G_2 at 4.5 h, whereas majority of wild-type cells are in G_1.

Mutations Are Clustered Mainly in the FAT and Kinase Domains of Tor2p. The above analysis identified single amino acid changes located in the FAT and kinase domains, pointing to the importance of these two domains. To further investigate the significance of these domains for Tor2p activation, we screened for additional mutations in the C-terminal half of Tor2p. The region containing either the FAT or the FRB, kinase, and FATC domains of the *tor2* gene in JUp1050 was randomly mutagenized, and 34 additional mutants that exhibited RIG were identified. Sequence analysis of these mutants revealed 17 additional single mutations at 15 positions.

Fig. 2*A* summarizes all of the activating mutations identified. Interestingly, we found that the mutations mainly were clustered in two regions: the N-terminal side of the FAT domain (cluster I) and the C-terminal portion of the kinase domain (cluster II). In addition, there were a few regions, notably at the C-terminal region of the FAT domain and the N-terminal region of the kinase domain, where additional mutations were identified. Fig. 2*B* shows sequence alignments of residues in which mutations

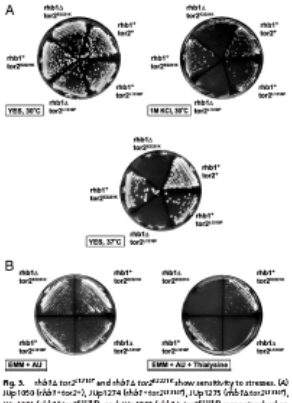

Fig. 3. *rhb1*Δ tor2^{L1310F} and *rhb1*Δ tor2^{E2221K} show sensitivity to stresses. (*A*) JUp1050 (*rhb1*$^{+}$ *tor2*$^{+}$), JUp1274 (*rhb1*$^{+}$ *tor2*^{L1310F}), JUp1275 (*rhb1*Δ *tor2*^{L1310F}), JUp1261 (*rhb1*$^{+}$ *tor2*^{E2221K}), and JUp1265 (*rhb1*Δ *tor2*^{E2221K}) were streaked on the indicated plates and incubated at the indicated temperatures. (*B*) JUp1265 (*rhb1*Δ *tor2*^{E2221K}) and JUp1275 (*rhb1*Δ *tor2*^{L1310F}) were transformed with either pREP41 (*rhb1*Δ) or pRPL-SpRhb1 (*rhb1*$^{+}$), and transformants were streaked onto the indicated plates and incubated at 30°C.

were found in clusters I and II. As can be seen, most of the mutations occur on residues that are perfectly conserved among TOR proteins from different organisms.

***rhb1*Δ tor2^{rig} Mutants Are Sensitive to Stress Conditions.** Analysis of a strain having a disruption of *rhb1* and carrying a *tor2*-activated (*tor2^{rig}*) mutation revealed that, although Tor2 mutants can bypass Rhb1p requirement for growth, they are incapable of bypassing other Rhb1p functions. Fig. 3*A* shows that the *rhb1*Δ strains that are viable because of the presence of either the *tor2^{L1310F}* or the *tor2^{E2221K}* mutation are sensitive to high-salt stress (1 M KCl) and high temperature (37°C). This sensitivity can be reversed by the introduction of *rhb1*$^{+}$, indicating that Rhb1p is involved in responding to these stresses in fission yeast.

We previously have shown that inhibition of Rhb1p causes hypersensitivities to toxic analogues of lysine (thialysine) and arginine (canavanine) (25, 46). In addition, loss of *tsc2* results in resistances to thialysine, canavanine, and ethionine (the toxic analog of methionine) (22, 23, 25). Examination of the *rhb1*Δ *tor2^{L1310F}* and *rhb1*Δ *tor2^{E2221K}* mutants on these toxic amino acid analogs showed that these double mutants are hypersensitive to thialysine, canavanine, and ethionine (Fig. 3*B* and data not shown). These sensitivities are reversed by reintroducing *rhb1*$^{+}$ into these cells. Thus, Rhb1p is required for the resistance to these amino acid analogs, and, because these cells carry activated Tor2p, this resistance likely is independent of Tor2p.

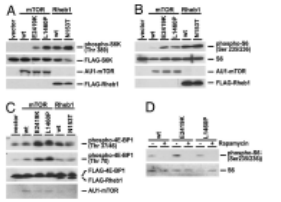

Fig. 4. mTOR^{L1460P} and mTOR^{E2419K} show constitutive activity. (*A–C*) HEK293 cells were transfected with pCDNA3 (vector), AU1-mTOR (wt, E2419K, or L1460P), or FLAG-Rheb1 (wt or N153T). To detect the phosphorylation of S6K or 4E-BP1, FLAG-S6K or FLAG-4E-BP1 was cotransfected. Cells then were serum-starved overnight and cultured in PBS for 1 h. The protein levels were detected by anti-FLAG (S6K, 4E-BP1, and Rheb), anti-AU1 (mTOR), or anti-S6 antibody. The phosphorylation levels of S6K, 4E-BP1, and S6 were detected by phospho-specific antibodies. (*D*) HEK293 cells were transfected with AU1-mTOR (wt, E2419K, or L1460P). Cells were serum-starved overnight and then treated with rapamycin (100 nM). The expression and phosphorylation levels of S6 were detected by specific antibodies. wt, wild type.

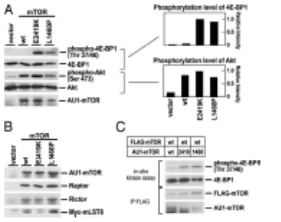

Fig. 5. Kinase activities of mTOR complexes. (*A*) HEK293 cells transfected with pCDNA3 (vector) or AU1-mTOR (wt, E2419K, or L1460P) were serum-starved overnight and then cultured in PBS for 1h. AU1-mTOR complexes then were immunoprecipitated with anti-AU1 antibody and used for *in vitro* kinase assays with 4E-BP1 and Akt as substrates. Phosphorylation of substrates was detected by use of the indicated phospho-specific antibodies. Levels of phosphorylation of substrates were quantitated relative to that by mTOR^{E2419K} and graphed. (*B*) HEK293 cells were transfected with pCDNA3 (vector) or AU1-mTOR (wt, E2419K, or L1460P) together with Myc-mLST8. Cells were serum-starved overnight and cultured for 1 h in PBS. AU1-mTOR complexes then were immunoprecipitated with anti-AU1 antibody. mTOR were detected by anti-AU1 antibody. mLST8 were detected by anti-Myc antibody. Raptor and Rictor were detected by specific antibodies. (*C*) HEK293 cells cotransfected with FLAG-mTOR and AU1-mTOR (wt, E2419K, or L1460P) were serum-starved overnight and cultured for 1 h in PBS. mTOR dimers were immunoprecipitated by using anti-FLAG antibody and used for *In vitro* kinase assays with 4E-BP1 as substrate. FLAG-mTOR and AU1-mTOR were detected by anti-FLAG and anti-AU1 antibodies, respectively. Phosphorylation of substrate was detected by use of anti-phospho-4E-BP1 (Thr 37/46).

Analogous Mutations in mTOR Confer Nutrient-Independent Activity. Because the mutations we identified occur mostly on residues that are conserved in higher eukaryotes, we asked whether these mutations would confer constitutive activation of mTOR. To examine this point, mTOR^{L1460P} and mTOR^{E2419K}, mutations that correspond to Tor2^{L1943P} and Tor2^{E2221K}, respectively, were constructed, and the regulation of their activities by nutrient signals in HEK293 cells was assessed. Consistent with previous reports (47, 48), mTOR activity as detected by the phosphorylation of S6K, S6, or 4EBP1 is inhibited when the cells are exposed to nutrient-starvation conditions (Fig. 4 *A–C*). This inhibition is overcome by overexpressing wild-type Rheb or a Rheb^{N153T} mutant, which is analogous to the hyperactive fission yeast Rhb1^{N153T} mutant that we previously have shown to be highly bound to GTP (Fig. 4 *A–C*) (25, 49). The activation seen by wild-type Rheb is likely attributable to Rheb being highly bound to GTP when transiently expressed (50).

We then examined the mTOR mutants. HEK293 cells were transfected with constructs that expressed either wild-type or the mutant mTOR (L1460P or E2419K) and then starved for nutrients. As can be seen in Fig. 4 *A*, *B*, and *C*, cells expressing mTOR^{L1460P} and mTOR^{E2419K} exhibit high phosphorylation of S6K, 4E-BP1, and S6 compared with wild-type mTOR, indicating that the mTOR mutants maintain activity even when the cells are starved for nutrients. However, these mTOR mutants retain sensitivity to inhibition by rapamycin (Fig. 4*D*). mTOR activity (S6 phosphorylation) was assessed in HEK293 cells expressing wild-type mTOR, mTOR^{L1460P}, or mTOR^{E2419K} and treated with either DMSO (−) or rapamycin (+). We found that both mTOR mutants were sensitive to rapamycin, similar to wild-type mTOR.

Constitutive activation of the mTOR mutants also can be examined by measuring *in vitro* kinase activities. The activities of the two mTORCs were assayed by using two different substrate proteins: mTORC1 activity was assayed by using 4E-BP1 (51, 52), whereas mTORC2 was assayed by using Akt as a substrate protein (53, 54). These complexes were immunoprecipitated from nutrient-starved cells by using AU1-tagged wild-type or mutant mTOR and activities assessed *in vitro* (Fig. 5*A*). Activity using Akt as the substrate was still retained with wild-type mTOR in HEK293 cells even under nutrient-starved conditions, and no change in this activity was observed when using the mutant mTOR. On the other hand, we found that the mTOR complex containing mTOR^{L1460P} or mTOR^{E2221K} exhibited significantly higher activity with 4E-BP1 as the substrate relative to wild-type mTOR. This activity was confirmed to be attributable to mTORC1, because an *in vitro* kinase assay using mTORC1 immunoprecipitated with anti-Raptor antibody showed similar results (SI Fig. 7). These results are consistent with our *in vivo* findings.

mTOR^{L1460P} and mTOR^{E2419K} Mutants Can Form mTORC1 and mTORC2 Complexes and an Active Heterodimer with Rhb1p-mTOR. We asked whether there were any alterations in the ability of the mTOR mutants to form mTORC1 and mTORC2. By using the AU1 tag on the expressed mTOR, mTOR complexes were immunoprecipitated from HEK293 cells under nutrient-starvation conditions, and levels of Raptor (mTORC1), Rictor (mTORC2), and mLST8 (mTORC1 and mTORC2) were assessed (Fig. 5*B*). We found that the mTOR mutants were able to bind similar amounts of these mTORC1 and mTORC2 components as was wild-type mTOR.

It has been reported that mTOR dimerizes via its N-terminal HEAT domains and that the dimeric mTORC1 is the major form that responds to insulin (36, 38). Because our mutations are not located in the HEAT domains, it is likely that wild-type and mutant mTOR would form a heterodimeric complex. To test whether this heterodimer exhibits constitutive activation of

mTOR function, we coexpressed FLAG-tagged wild-type and AU1-tagged wild-type or mutant mTOR proteins in HEK293 cells. The cells were starved for nutrients, and the complexes were isolated by immunoprecipitation using anti-FLAG antibody. We found similar amounts of AU1-tagged wild-type mTOR and the AU1-tagged mutant mTOR in the immune complex, indicating that mutant mTOR was able to form a heterodimer with wild-type mTOR (Fig. 5*C*). Furthermore, when these complexes were assayed for activity by using 4E-BP1 as a substrate, we found that, although the wild-type/wild-type dimers were inactive, the wild-type/mutant heterodimers exhibited *in vitro* kinase activity (Fig. 5*C*).

Discussion

Accumulating evidence in fission yeast provides strong support for the idea that Rhb1p is an activator of Tor2p. We have reported that Rhb1p associates with Tor2p (25). We and others also recently have shown that, in fission yeast, Tor2p complexes with Mip1p (the fission yeast Raptor homolog), likely forming a SpTORC1 (44, 45). Furthermore, shutting down Tor2p results in small rounded cells arrested in G_1, reminiscent of inhibiting Rhb1p (44, 45). In addition, inhibition of Tor2p in homothallic cells induces mating and sexual development (44, 45). Now, we show that, opposite to inhibiting Tor2p function, an activating mutation in Tor2p can confer R1G, delayed response to nitrogen starvation, and decreased sexual development.

This report provides evidence for a large number of single amino acid changes that confer constitutive activation of Tor2p. Importantly, many of these mutations occur in residues that are highly conserved in TOR proteins, and mTOR carrying analogous mutations exhibit nutrient-independent mTORC1 activation. An exciting finding of our study is that the activating mutations are clustered within two domains of TOR: the N-terminal region of the FAT domain (cluster I) and the C-terminal region of the kinase domain (cluster II). Interestingly, the cluster in the kinase domain is just adjacent to the repressor domain (2430–2450 of mTOR; Fig. 2*B*); deletion of this region results in activation of mTOR (39, 40). Indeed, the analogous position (2431 of mTOR) for one of the activated fission yeast Tor2 mutants, L2233H, is found just inside this repressor domain. This close proximity of the repressor domain to cluster II possibly indicates that these mutations may activate mTOR via a similar mechanism. The deletion of the repressor domain initially was thought to activate mTOR, in part by removing an Akt phosphorylation site (S2448; Fig. 2*B*); however, mutating this site, which later was shown to be an S6K phosphorylation site (42, 43), to alanine does not significantly alter mTOR activity (39). Furthermore, mutating the AMPK phosphorylation site (T2446; Fig. 2*B*) to alanine did not alter mTOR activity (39, 41). These phosphorylation sites also are not conserved in fission yeast Tor2p. Perhaps this region is involved in interacting with an unknown inhibitory factor. In addition to cluster II, mutations were found in the N-terminal region of the kinase domain. Interestingly, Rheb is reported to interact with the N-terminal half of the mTOR kinase domain (29). It is possible that these mutations in the kinase domain mimic the effects of Rheb binding, which may interfere with this inhibitory factor. Further experiments are needed to address the consequences of these mutations. A recent report in budding yeast identified mutations in the FRB that exhibit increased association with KOG1p (budding yeast Raptor homolog) and increased activity to *Saccharomyces cerevisiae* TOR1p (55). Although the FRB region was included in our mutagenesis, we did not identify any activating mutation in this region.

The activation of Tor2p results in a delay in the nitrogen-starvation response. However, this response is not a complete block. Furthermore, we find that *rhb1*Δ *tor2*^{ts} strains also are able to respond to nitrogen starvation and undergo sexual differentiation (SI Fig. 8). The observation that these strains still are able to respond to nitrogen starvation in the absence of Rhb1p raises the possibility that Tor2p also is regulated by an Rhb1p-independent mechanism. Further analysis of *rhb1*Δ *tor2*^{ts} cells also revealed possible involvement of Rhb1p in stress response. These mutants are sensitive to stresses such as high salt (1 M KCl) and high temperatures (37°C). Although the mechanisms for these phenotypes need further investigation, it is of interest to note that *tor1*Δ mutants also exhibit these phenotypes. Another phenotype of the *rhb1*Δ *tor2*^{ts} mutants is that they are hypersensitive to toxic amino acids analogs. It is possible that these sensitivities are a result of increased amino acid uptake because we previously have shown that decreases in Rhb1p function lead to increased uptake of arginine and hypersensitivity to canavanine (46).

We have succeeded in identifying mTOR mutants that are active independent of nutrients. These mutants provide valuable reagents to further examine the biological consequences of mTOR activation. By introducing these mutants in HEK293 cells, we have shown that they confer nutrient-independent mTORC1 signaling. Additional experiments may shed light onto the role of mTOR activation in growth. Introducing these mutants in HEK293 as well as other untransformed cell lines also may provide insight into mTOR activation in transformation. Investigating mTOR activation in whole animals also is of importance. These mutants can be introduced into mice, and such animals can be studied further for roles of mTOR in development as well as propensity for tumors.

Activation of the mTOR pathway has been implicated in a number of human diseases associated with benign tumors, such as hamartomas (56). Our demonstration that mTOR can be activated by single amino acid changes raises an interesting possibility that mTOR mutations may be found in tumor samples. This idea is supported further by our finding that our mutant mTOR is active even when in a heterodimer with the wild-type protein. Because our study points to two hot spots in the TOR protein, searches for mTOR mutants may be focused on these two regions. The results obtained from these studies should have significant implication for our understanding of human diseases arising from the activation of the TSC/Rheb/mTOR signaling pathway.

Materials and Methods

Screens for RIGs and Activated Tor2p. *RIG screen.* The scheme for the RIG screen is shown in Fig. 1. JUp1050 strain (3×10^8 cells) was mutagenized with methyl-nitro-nitrosoguanidine (Sigma, St. Louis, MO) and recovered in rich yeast extract with supplements (YES) media overnight. Cells then were plated onto Edinburgh minimal medium supplemented with adenine (225 mg/l), leucine (225 mg/l), uracil (50 mg/l), and FOA (1 g/l). Plates were replica-plated once to eliminate background. Of ≈3,000 colonies, 241 clones were isolated and tested for absence of *rhb1*$^+$ by colony PCR. One clone was isolated that had lost the *rhb1*$^+$ plasmid.

Screen for tor2 mutants exhibiting decreased mating. Random mutations were introduced into the *tor2* gene by PCR (57). Linear DNA fragments carrying mutagenized *tor2* alleles were transformed into JV530, a homothallic strain in which the endogenous *tor2* gene is disrupted with a kan^r cassette and whose growth is maintained by a multicopy plasmid pREP42-tor2. To obtain integrants of functional *tor2* alleles, transformants that were resistant to FOA (indicating loss of *ura4*$^+$-based pREP42-tor2) and kan^r were screened at 26.5°C. From a *tor2* mutant library thus constructed, we screened for sterile clones; each strain was grown to a colony on SSA plate (see *SI Materials and Methods*) and stained with iodine vapor after incubation for 4 days at 30°C. Unstained colonies were isolated and examined microscopically for sterility.

Screen for additional FAT and kinase mutants. Additional FAT and kinase domain mutations were identified by screening libraries based on pUG6-tor2-CTL in which the region containing the FAT domain or the FRB, kinase, and FATC domains were randomly mutagenized by using the GeneMorph II Random Mutagenesis Kit (Stratagene, La Jolla, CA). The libraries were digested with BamHI, and the linearized plasmids were integrated into JUp1050. The resulting transformants initially were selected on plates containing G418 (200 μg/l) for integration of plasmid and then on FOA for the RIG phenotype as before. Then, 8,400 and 12,000 integrants were screened from the FAT domain library and the FRB-kinase-FATC domain library, respectively. In both cases, 17 clones were isolated. The regions that were mutagenized were PCR-amplified from genomic preps and sequenced to identify the mutations.

Mammalian Cell Culture and Transfection. HEK293 cells were cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C and 5% CO_2. Transfections were performed by using Polyfect (Qiagen, Valencia, CA) according to the manufacturer's instructions. To assess the activity of mTOR mutants, cells were serum-starved in DMEM supplemented with 0.1% BSA overnight and then cultured in PBS for 1 h. For rapamycin treatment, cells were treated with 100 nM rapamycin for 1 h after serum starvation. These cells were lysed, and proteins were analyzed by Western blotting analysis.

Immunoprecipitation and *In Vitro* Kinase Assay. Cells were lysed with buffer A [20 mM Tris·HCl (pH 7.5), 150 mM NaCl, 0.5% CHAPS, 1 mM $MgCl_2$ and 1 mM EDTA]. The supernatant from the centrifugation at 20,000 × *g* for 15 min was incubated with anti-AU1 antibody (Covance, Berkeley, CA) and protein G-Sepharose 4FF beads (Amersham Biosciences, Piscataway, NJ) at 4°C for 2 h. Immunoprecipitates were washed three times with buffer A. For *in vitro* kinase assay, immunoprecipitates were incubated in kinase buffer [100 mM Tris·HCl (pH 7.5), 50 mM $MgCl_2$, and 1 mM ATP] containing 0.5 μg GST-4E-BP1 or Akt for 30 min at 37°C. Samples were boiled in SDS sample buffer [3% SDS, 5% glycerol, 62 mM Tris·HCl (pH 6.7)], and proteins were analyzed by Western blotting analysis.

SI. Additional information regarding yeast strains, media and manipulations, cell cycle and size analysis, plasmid constructs, and antibodies and reagents is provided as *SI Materials and Methods*. A list of strains used in this study is provided in SI Table 1.

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